Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/043679

International filing date: 23 December 2004 (23.12.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/532,089
Filing date: 23 December 2003 (23.12.2003)

Date of receipt at the International Bureau: 11 February 2005 (11.02.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

1280844

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

February 03, 2005

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/532,089*
FILING DATE: *December 23, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/43679*

Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

PTO/SB/16 (10-01)
Approved for use through10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a requ st f r filing a PR[illegible]NT under 37 CFR 1.53(c).

Express Mail Lab l N . ER673421955US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Xing-Xiang	Li	Vienna, Virginia

☑ *Additional inventors are being named on the* 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Methods for improving the sensitivity of analyte detection

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☐ Customer Number [ ] → *Place Customer Number Bar Code Label here*

Type Customer Number here

OR

☐ Firm *or* Individual Name	Xing-Xiang Li				
Address	9023 Edgepark Rd				
Address					
City	Vienna	State	VA	ZIP	22182
Country	U.S.A	Telephone	(703)821-1612	Fax	(703)821-0299

ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 6
☐ Drawing(s) *Number of Sheets*
☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☑ Other (specify) Self adressed card

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.
☐ A check or money order is enclosed to cover the filing fees
☐ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: [ ]
☑ Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($): $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.
☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE [signature]

Date 12-22-03

TYPED or PRINTED NAME Xing-Xiang Li

REGISTRATION NO. *(if appropriate)*

Docket Number:

TELEPHONE (703)821-1612

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

PROVISIONAL APPLICATION COVER SHEET
Additional Page

PTO/SB/16 (02-01)
Approved for use through 10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Docket Number	

INVENTOR(S)/APPLICANT(S)

Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
Tianxin	Wang	9768 Early Spring Way, Columbia, MD 21046

Number 1 of 1

WARNING: Information on this form may become public. Credit card information should not be included on this f rm. Pr vide cr dit card informati n and auth rizati n on PTO-2038.

Methods for Improving the Sensitivity of Analyte Detection

Current invention relates to an improvement of methods for analyte detection using chemical markers or the like.

Specific detection of analytes using markers such as fluorescent compounds is a common practice in research, biotechnology and medical diagnostic arenas. In such detection methods, a ligand specific for an analyte (e.g., nucleic acids, antigen or antibody) is directly or indirectly labeled with one or more markers. Marked ligands are then allowed to bind to the analyte under conditions that favor specific binding. After removal of unbound ligand through washing, the bound markers are often measured using an instrument capable of detecting the markers.

In order to increase the sensitivity, it is desirable to increase the number of marker molecules per bound ligand. For example, a method with a 10:1 molar ratio of marker to bound ligand will likely be more sensitive than one with 1:1 ratio. A carrier can be used to increase such molar ratio. A carrier can be a microsphere, which contains multiple sites to which multiple fluorescent molecules and ligands can be attached. Alternatively, a great number of fluorescent molecules can be encapsulated within microspheres whereas the ligands can be attached to the surface of the particles. When a ligand binds to an analyte molecule, multiple marker molecules bind to the analyte thereby increasing the sensitivity. The ligand-analyte-marker complex can then be captured on to a solid surface to which a second ligand specific for the analyte is attached. This method of detection is often known as signal amplification. The higher the ratio of marker to bound ligand, the greater the signal is normally amplified.

Problems may arise, however, when a carrier contains too many markers in close proximity. For example, fluorescent compounds tend to quench one another when they are confined in a close proximity resulting in decreasing of fluorescent signal. When encapsulated in microparticles or the like, fluorescent markers may also quench one another if there are too many markers in a microparticle. For those markers that require no excitation light (e.g., chemiluminescent compounds, electrochemolumincescent compound and enzymes), problems may still exist in that they have to be in direct contact with trigger conditions such as reagents for chemoluminescent compounds or electricity for electrochemolumincescent compounds. The applicants now invent methods that solve these problems.

The key aspect of the current invention is to release the markers from the carriers before detection. By freeing the markers from the carriers, one solves the problems of quenching or accessibility to trigger reagents or both and allows high load pf marker.

In general, markers are coupled to or encapsulated in a carrier, to which analyte-specific ligands are also attached. Detection of an analyte involves the binding of *multiple markers-carrier-ligand* complex to the analyte, which is then immobilized on a solid surface (e.g., magnetic particles) to which a second analyte specific ligand is attached.

Alternatively, the analyte is first immobilized onto a solid surface followed by binding of markers-carrier-ligand complex. After removal of unbound marker-carrier-ligand complex normally through washing, the bound markers are released from the carrier and then detected with desired means such as fluorometer for fluorescent markers.

A wide range of other well known methods can be used to separate the markers-carrier-ligand complex bound with the analyte, such as varieties of chromatography methods, micro sphere techniques, magnetic particle techniques, flow cytometer, lateral flow techniques, solid phase immobilization, solid phase extraction, centrifuge, electrophoresis, filtration and etc. The multiple marker molecules can be released later using physical or chemical means from the carrier for detection.

The general procedure is depicted in Figure 1.

Appropriate markers include, but are not limited to, fluorescent compounds (such as fluorescein) , quantum dots, or rare earth elements (e.g., Europium, could be in the form of salt, chelate, oxide, metal et ac.), chemoluminescent compounds (such as acridinium derivatives), electrochemoluminescent compounds (such as rare earth elements), dyes, or the precursor or derivatives of above markers and enzymes or its inhibitors or its activators. A skilled person can easily find many suitable markers from textbook or scientific journal papers or other information source. Appropriate carriers include, but are not limited to, polymers such as linear polymers (e.g., poly lysine, poly acrylic acid, peptides and proteins) and highly branched macromolecules (e.g., dendrimers), and microparticles (such as microspheres, micro beads, nano particles, microcapsules, liposomes) or the like. The carriers normally contain multiple functional groups such as carboxyl group and primary amines, which permit the labeling of multiple markers and ligands. A skilled person can find many suitable labeling chemistry from textbook or scientific journal papers or other information source. Preferably, ligands are coupled to the carrier covalently and in a fashion that permits their contact with the analyte in a solution or on solid surface. For example, ligands are coupled to the surface of microspheres.

In certain embodiment, the markers are coupled to the carriers using cleavable linkers, which include, but are not limited to, photo labile bond, chemical sensitive bond, pH sensitive bond, and heat sensitive bond. The marker molecules can be released using varieties of methods such as oxidation, reduction, acid-labile, base labile, enzymatic, electrochemical, heat and photo labile methods, dissolution and etc.

Cleavable linkers may also include noncovalent bonds, which include, but are not limited to, hydrogen bonds (e.g., those in nucleic acid base pairing), ion paring, biotin-streptavidin interaction, and chelating and etc. Under normal assay or storage conditions, the linker between the markers and carriers is stable, which permits normal assay procedures such as washing. When unbound markers are removed, bound markers are cleaved or dissociated from the carriers with desired means. For example, one can use a UV light to cleave UV light sensitive photo labile bond that joints the markers and carriers thereby freeing the markers to the medium. Detection is then carried out.

In other embodiment, markers are encapsulated in carriers such as microparticles or the like. For these carriers, the markers are released through physical or chemical means, which result in swelling, dissolution, or partial disintegration (e.g. perforation) of microparticles or the like. The physical means include, but are not limited to, heat and pH condition and mechanical force. The chemical means include, but are not limited to, organic solvents and acidic or alkaline conditions. For example, one can use DMSO (dimethyl sulfoxide) at appropriate concentration to dissolve polystyrene microspheres to free encapsulated markers for detection.

Example 1

Figure 1 below illustrates an example according to the teaching in the current invention. The assay is designed to detect certain antigen 5 in the sample containing other components 4. Antibody specific for antigen 5 is immobilized on the surface of a solid phase support such as the surface of a microwell plate well 1. A sample diluted in a desired buffer is added to the wells. After incubation at desired conditions (temperature and duration), the sample solution is removed and the well is washed a desired number of times. If present in the sample, antigen 5 will be retained on the well wall. Microsphere 3 coupled with multiple fluorescent molecules 2 and antibody specific to antigen 5 are then added to the well. In this example, the fluorescent molecules are coupled to the particles through a linker containing a disulfide bond (e.g., dithiopropionate), which is thiol-cleavable. Upon binding, the unbound microspheres are removed from the well through washing.

Fluorescent molecules 2 are cleaved from the microspheres using a reducing agent such as beta-mercaptoethanol. The released fluorescent molecules 2 can be readily detected using a fluorometer. The fluorescence intensity is proportional to amounts of bound microspheres, which are in turn proportional to the amount of antigen 5 in the sample.

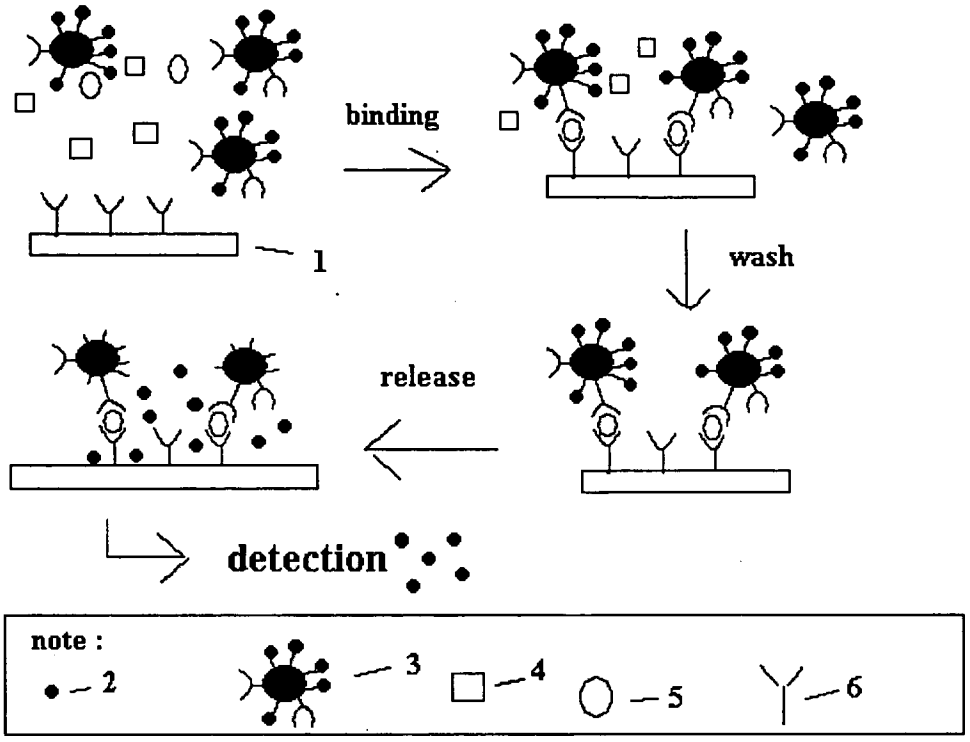


Figure 1: an illustration for example 1.

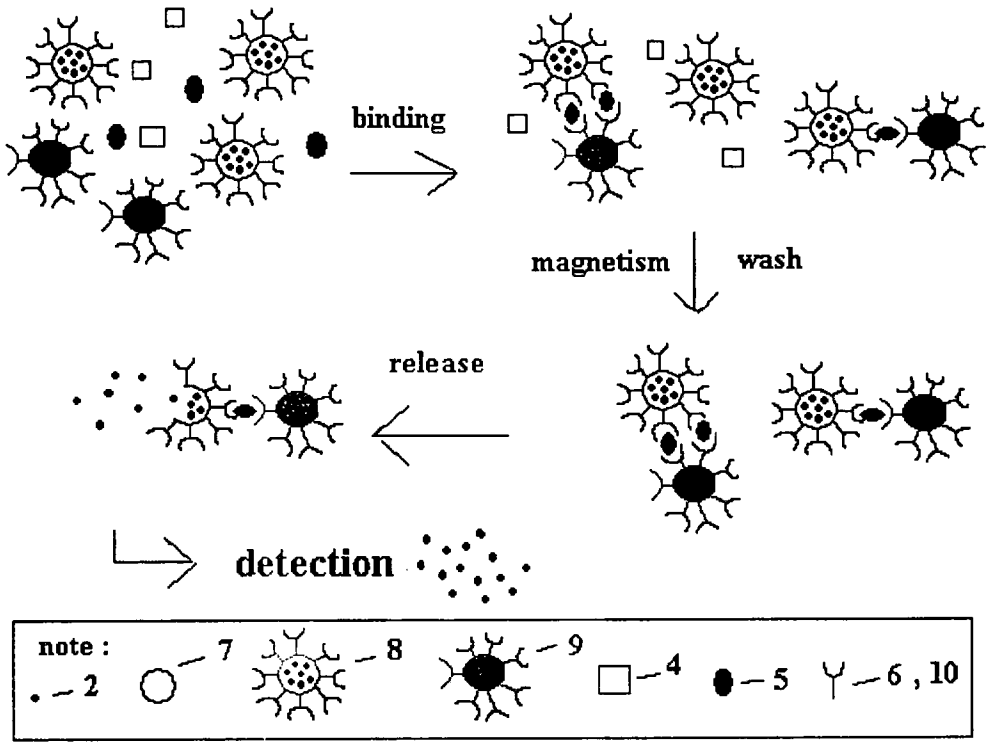
Example 2

In this example, fluorescent markers 2 are encapsulated in polystyrene microspheres 7. Antibodies 6 specific for antigen 5 are covalently labeled to the surface of microspheres 7, thereby creating microspheres 8 encapsulated with fluorescent markers 2 and antibodies 6.

In this example, the solid surface is provided with magnetic particles 9, which are coated with the same antibodies 6 or, preferably, different antibodies 10 specific for a distinct epitope of antigen 5.

To detect antigen 5 in a sample, the magnetic particles 9 and microsphere 8 are first mixed at appropriate concentrations in a desired buffer. The sample is added to the particle mixture with or without dilution as desired. After incubation, the presence of antigen 5 causes agglutination between magnetic particles and 9 microspheres 8. Microshperes bound to magnetic particles is then separated from unbound ones and other analyte 4 using a magnet. After washing to reduce the background and non-specific binding, a desired amount of DMSO is added to the particles. DMSO dissolves (partially or completely) or swell microspheres 8 thereby releasing the fluorescent markers, which are then measured using an instrument such as fluorometer. If several different fluorescent markers are encapsulated in distinct microspheres targeting different analytes, multiple analytes detection can be achieved simultaneously due to the spectral difference of these markers. In addition, two or more fluorescent markers allow the use of internal control for an assay. Obviously, different marker types (e.g., fluorescence vs.

chemoluminescence) or same marker type with distinct kinetics (e.g., flush light vs. glow light) may also be used in a single assay to detect multiple analytes or function as an internal control.



If the microparticle is porous, and the marker molecules are soluble in certain solvent, the certain solvents can also be used to release the marker molecules inside. The marker molecules can also be embedded in the microsphere and coupled to the inside of the microsphere via cleavable linker, in this case dissolving/swelling solvent plus cleaving means is needed. However, in some cases the marker groups such as the chemiluminescent agents or fluorescent agents can be coupled to the inside of microsphere via non-cleavable linker since they can still give detectable signal (such as light) in the coupled state after being dissolved or swelled.

It is understood that the carrier can also be a soluble polymer such as poly lysine, poly acrylic acid, dendrimer, and etc having multiple functional groups to couple with multiple copies of releasable markers such as Eu ion.

Alternatively, the marker group and the affinity group can be coupled together to form a complex and the complex is coupled to the carrier. The marker can later be released for detection.

The ligands on one copy carrier and the purification surface (such as the magnetic beads) could contain a group of ligands that targeting different regions of one analyte to increase the binding. For example, multiple pairs of affinity DNA group targeting the different

regions of one HIV RNA could be coupled to the magnetic beads and the carrier for detection of HIV virus. In another case, if the ligands on one copy carrier are specific to a groups of analytes (such as a mixture of several different antibodies), it can be used to detect this analyte group instead of one analyte.

It is preferred that the ligands on the carrier (e.g. microparticles) are different from those on capture or purification surface (such as the magnetic beads), which improves binding efficiency. For example, one or more distinct affinity probes for HIV-1 RNA can be coupled to a carrier and magnetic for detection of HIV-1 virus.

Similarly, more than one antigens or antibodies can be used in the example for the detection of a pathogen.